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Production and characterization of amphihaploid hybrids between *Nicotiana wuttkei* Clarkson et Symon and *N. tabacum* L

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Abstract *Nicotiana wuttkei* Clarkson and Symon discovered in the 1990s in Australia may be of potential interest to breeders as it carries resistance to *Peronospora hyoscyami* de Bary. The crossability between *N. wuttkei* ($2n = 4x = 32$) and three *N. tabacum* ($2n = 4x = 48$) cultivars ('Puławski 66', 'Wiślica' and 'TN 90') and the morphology and cytology of their amphihaploid hybrids ($2n = 4x = 40$) were studied. Seeds were produced only when *N. wuttkei* was used as the maternal parent, but under normal germination all seedlings died. Viable F_1 hybrids of *N. wuttkei* × *N. tabacum* cv. 'Puławski' and *N. wuttkei* × *N. tabacum* cv. 'Wiślica' were obtained only by in vitro cotyledon culture. The amphihaploid plants were intermediate between the parents for most morphological traits. In 46.4% of the PMC's, only univalents were present. The remainder of the cells had 1–5 bivalents and 1–2 trivalents. In spite of a detectable frequency of monads (2.6%), dyads (2.6%) and triads (4.5%), the hybrids were self and cross sterile.

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Introduction

The advances in genetic engineering have to some extent eclipsed the importance of wide hybridization as a tool for gene transfer from phylogenetically distant sources, but this method may still be an asset in cultivar development especially when the use of transgenic methods is to be avoided. Since the pioneering work by Holmes (1938) wide crosses and synthetic amphiploids have played an important role in introgresssions of desirable traits from related species into cultivated tobacco. A considerable part of that effort was directed towards resistance to blue mould caused by *Peronospora hyoscyami* de Bary (*Peronospora tabacina* Adam), a devastating leaf disease now spread all over the world but originally endemic to Australia. At least a few native wild Australian tobaccos have evolved resistance to the blue mold pathogen. Among them, *Nicotiana debneyi* (Clayton 1968), *N. goodspeedii, N. velutina* (Wark 1970), *N. excelsior* and *N. exigua* (Gillham et al. 1977) have been reported as sources of resistance in developing blue-mold resistant tobacco cultivars.

Another potential candidate for possible resistance transfer to cultivated tobacco is *N. wuttkei,* a relatively recently discovered wild tobacco (Clarkson

and Symon 1991), and yet another Australian species resistant to *Peronospora hyoscyami* (Laskowska and Berbeć 2003). Its originally reported chromosome number of 28 was subsequently corrected to 32 (Laskowska and Berbeć 2003). It is probably a natural allopolyploid of polyphyletic origin, as are other species of the Australian section *Suaveolentes* (Chase et al. 2003) and, like them, is a distant relative of the cultivated allopolyploid *N. tabacum* ($2n = 4x = 48$).

Although blue mold resistant cultivars of tobacco are widely used all over the world, diversification of available sources of resistance may be a worthwhile effort. The blue mold pathogen is notorious for mutations and the ability to develop new races (Masiak 1982; Zipper et al. 2009) and, according to the recent molecular studies (Milla et al. 2005), all important resistant cultivars probably derive their resistance from a single source (*N. debneyi*) even if previously reported otherwise. The hybridization of *N. wuttkei* with *N. tabacum* may also be important for basic studies in plant biology, gaining increased understanding of phylogenetic relationships among species, and for investigating aspects of evolution of the genus *Nicotiana*.

The present study reports on the production, morphology, meiotic behaviour and fertility of the amphihaploid hybrids between *N. wuttkei* and *N. tabacum.*

Materials and methods

Plant materials

Nicotiana wuttkei Clarkson and Symon ($2n = 4x = 32$) and three diverse cultivars of *N. tabacum* L. ($2n = 4x = 48$): 'Puławski 66', 'Wiślica', and 'TN 90' were used in the interspecific hybridization. The F_1 hybrids containing a haploid set of chromosomes from each parental species ($2n = 4x = 40$) are called amphihaploids (Goodspeed 1954; Chaplin and Mann 1961).

Hybrid production

Reciprocal crosses were made in the greenhouse of the Institute of Soil Science and Plant Cultivation State Research Institute, Puławy, Poland in 2000. The crossability of *N. wuttkei* with *N. tabacum* cultivars and that of their hybrids was determined based on the number of pollinated flowers, the number of seeds obtained and the percentage of germinating seeds. Seeds of the resulted F_1 generation were germinated in vitro. Seeds were sterilized with 10% hydrogen hyperoxide for 20 min and placed on the LS medium (Linsmaier and Skoog 1965). Cotyledons from germinated seeds were cut into 2–3 segments each and placed on the Lloyds medium (Lloyd 1975). Regenerated shoots were rooted on the LS medium supplemented with 0.2 mg/l IAA and 0.2 mg/l NAA and transferred to pots with soil. The viable F_1 amphihaploid hybrids were grown to maturity in a greenhouse in 2001.

Cytological studies

Mitotic chromosome counts were performed on juvenile corollas of greenhouse-grown plants using the method of Burns (1964). Corolla fragments were pretreated for 5 h in a 0.44% solution of 8-oxyquinoline with saturated maltose solution, added just before using (0.05 ml maltose per 3 ml 8-oxyquinoline). The preatreated material was fixed in the Carnoy solution (ethanol, chloroform and acetic acid—6:3:1) and acetocarmine (1.5%)—stained preparations were made. Chromosome numbers were determined for ten cells of each obtained plant.

For observations of meiotic configurations, the flower buds at the appropriate stage (determined by a preliminary microscopic examination of one of the five anthers in a bud) were fixed in the Carnoy's fluid for 24 h and stored in 70% ethanol. Anthers were squashed in a drop of 1.5% acetocarmine. The meiosis tests included observations of chromosome pairing at metaphase I, meiotic irregularities at further stages (chromatin bridges, laggards) and number of microspores and micronuclei produced at the tetrad phase. On the basis of chromosome pairing data, the means and ranges for the numbers of bivalents, univalents and trivalents were estimated. Observations of meiotic configurations were performed in approximately 100 pollen mother cells (PMCs) prepared from anthers taken from four plants of each genotype.

Pollen viability was assessed with acetocarmine in at least 1,000 pollen grains from each plant.

Results

Crossability

From 307 *N. tabacum* flowers pollinated with *N. wuttkei* pollen, not a single seed capsule developed. Hybrid seeds were obtained only when *N. wuttkei* was used as the maternal parent. Their germination capacity was quite high and amounted to 66.9, 80.0 and 67.2% in crosses with *N. tabacum* cv. 'Puławski', 'Wiślica', and 'TN 90', respectively. Already at the cotyledon stage hybrid seedlings exhibited lethal symptoms, such as browning of the hypocotyl and of the roots, and all died when grown directly in the soil. When grown in vitro, nearly 99 percent of the seedlings also failed to survive beyond the cotyledon stage and only 11 of the 310 germinated hybrid seeds (3.55%) produced viable hybrids from the *N. wuttkei* × *N. tabacum* cv. Puławski combination; in the *N. wuttkei* × *N. tabacum* cv. Wiślica combination, of the 2,333 seeds only 27 (1.16%) produced viable hybrids (Table 1), for the total of 38 F_1 hybrids. These plants had good vigour after transplanting into soil and developed to flowering.

Morphology and cytology of amphihaploid hybrids

Twenty-six plants resulting from the *N. wuttkei* × *N. tabacum* cross and regenerated from in vitro-cultured cotyledons had 40 chromosomes in their somatic cells. The remaining 12 plants had double the number of chromosomes (80). There plants will be described in a separate report.

Morphologically, the 40-chromosome plants were intermediate between their parents (Fig. 1a, b, c). Unlike in *N. wuttkei*, lower leaves did not form a basal rosette (Fig. 1b). The hybrids were as tall as or taller than the *N. tabacum* parent; the flowers were intermediate in shape and size (Fig. 1d) and pink in color, a trait of the *N. tabacum* parents used in this study. The leaves were more like those of the *N. tabacum* than those of *N. wuttkei* both in shape and in size (Fig. 1e).

The chromosome associations were observed in metaphase I of the *N. wuttkei* × *N. tabacum* hybrids (Table 2), and some representative cells are shown in Fig. 2. Chromosome pairing varied from cell to cell, but there were no significant differences between the hybrids with regard to the calculated means of metaphase I associations per PMC. Meiosis was largely asynaptic. Of the 653 PMCs analyzed, 303 (46.4%) had all 40 chromosomes as univalents. As it is usually the case with univalents, they failed to form a metaphase plate and were scattered throughout the volume of the PMC (Fig. 2a). The remainder of the cells had between 1 and 5 bivalents and 1–2 trivalents, and the rest were univalent. One (Fig. 2b), two (Fig. 2c, d), three, four (Fig. 2e) and five (Fig. 2f) bivalents were observed in 31.4, 14.1, 7.2, 0.6 and 0.2% of the PMCs, respectively. The modal number of bivalents was 0. The great majority of bivalents observed in metaphase I were open, hence they had one chiasma each (Fig. 2b, c, d, e, f). Some chromosomes in bivalents appeared to be bound by very thin chromatin strands and tended to separate precociously (Fig. 2e). Trivalents (Fig. 2e) were recorded in a few PMCs.

Disjunctional abnormalities observed in later meiotic stages included chromatin bridges and lagging chromosomes, restitution nuclei and multipolar division spindles. The numbers of microspores at the tetrad stage are listed in Table 3 and in Fig. 3.

Table 1 Crossability between *Nicotiana tabacum* and *N. wuttkei*

Cross	Number of			Percent of	
	Flowers pollinated	Seed capsules obtained	Seeds obtained	Seed germinated	Viable hybrids[a]
N. tabacum × *N. wuttkei*	307	0	0	–	–
N. wuttkei × *N. tabacum*					
N. wuttkei × Puławski 66	55	50	463	66.9	3.55
N. wuttkei × Wiślica	50	30	2916	80.0	1.16
N. wuttkei × TN 90	75	55	2115	67.2	0
Total	180	135	5494	72.6	0.95

[a] Obtained by in vitro cotyledon culture, calculated as (the number of viable hybrids/the number of hybrids used for cotyledon culture) × 100

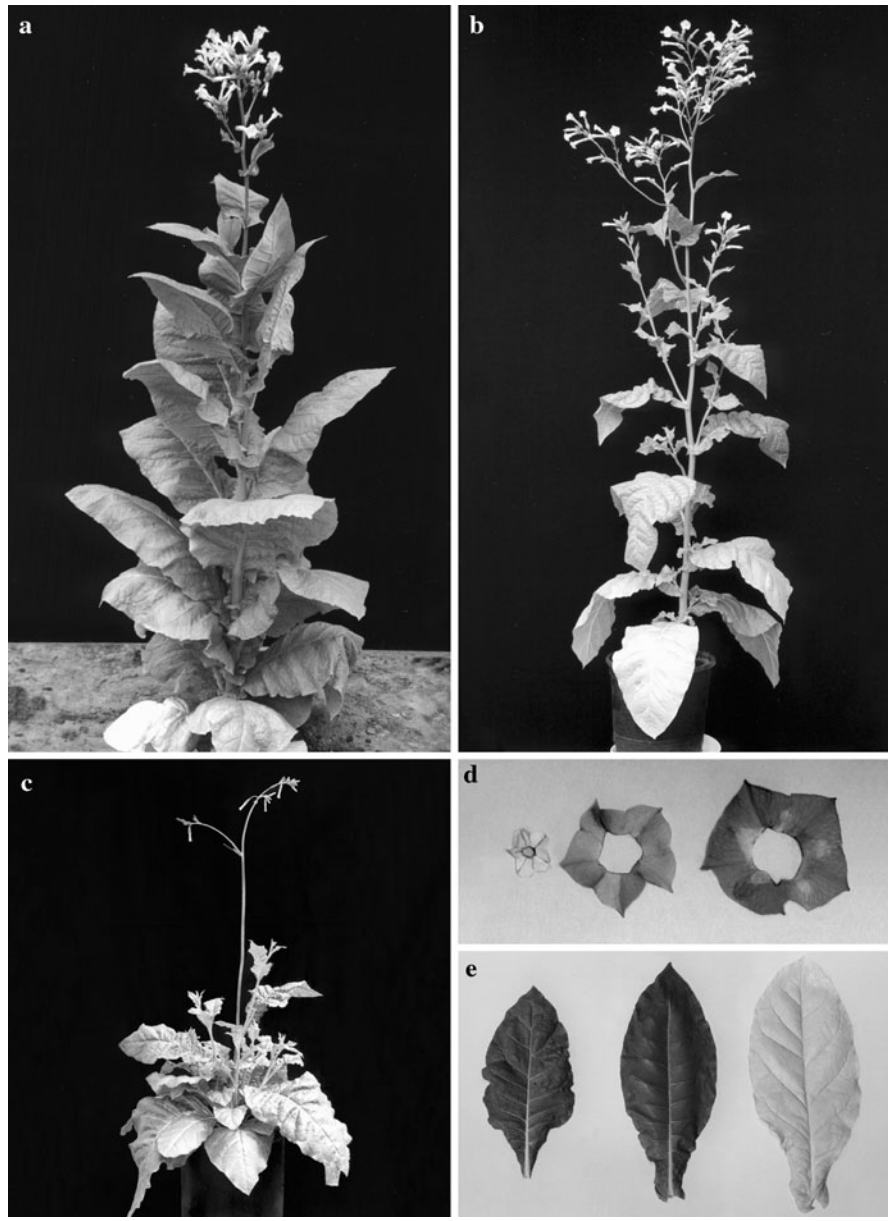

Fig. 1 Comparison of morphological attributes of *N. tabacum* cv. Wiślica, *N. wuttkei* and their F_1 hybrid. Plant habit: *N. tabacum* cv. Wiślica (**a**), F_1 hybrid (**b**), *N. wuttkei* (**c**), corollas (**d**) and leaves (**e**) of *N. wuttkei*, F_1 hybrid and *N. tabacum* cv. Wiślica (*left* to *right*)

Tetrads were observed in the 82.3% of 468 PMCs examined (Fig. 3c) and monads (2.6%), dyads (3.8%), tryads (4.5%) and pentads (6.8%) in the remaining part of PMCs (Fig. 3a, b, f). Laggards not included in daughter nuclei formed micronuclei in 10.0% of all PMCs observed (Fig. 3d, e). The amphihaploid hybrids did not produce viable pollen and were self and cross sterile.

Discussion

Wide crosses have played an important role in the study of phylogenetic relationships among species and in widening the genetic basis of tobacco including the transferr of disease resistance traits to commercial *N. tabacum* cultivars. *N. wuttkei* is a newly discovered species (Clarkson and Symon

Table 2 Metaphase I chromosome pairing in the amphihaploid hybrid *Nicotiana wuttkei* × *N. tabacum*

Hybrid	Number of PMCs observed	Chromosome pairing per PMCs		
		Univalents	Bivalents	Trivalents
N. wuttkei × *N. tabacum* cv. Puławski	292	38.38 ± 0.114[a] (29–40)[b]	0.79 ± 0.055 (0–4)	0.014 ± 0.008 (0–2)
N. wuttkei × *N. tabacum* cv. Wiślica	361	38.21 ± 0.106 (30–40)	0.89 ± 0.052 (0–5)	0.003 ± 0.003 (0–1)
Total	653	38.26 ± 0.078 (29–40)	0.84 ± 0.038 (0–5)	0.008 ± 0.004 (0–2)

[a] Mean ± SE

[b] Range

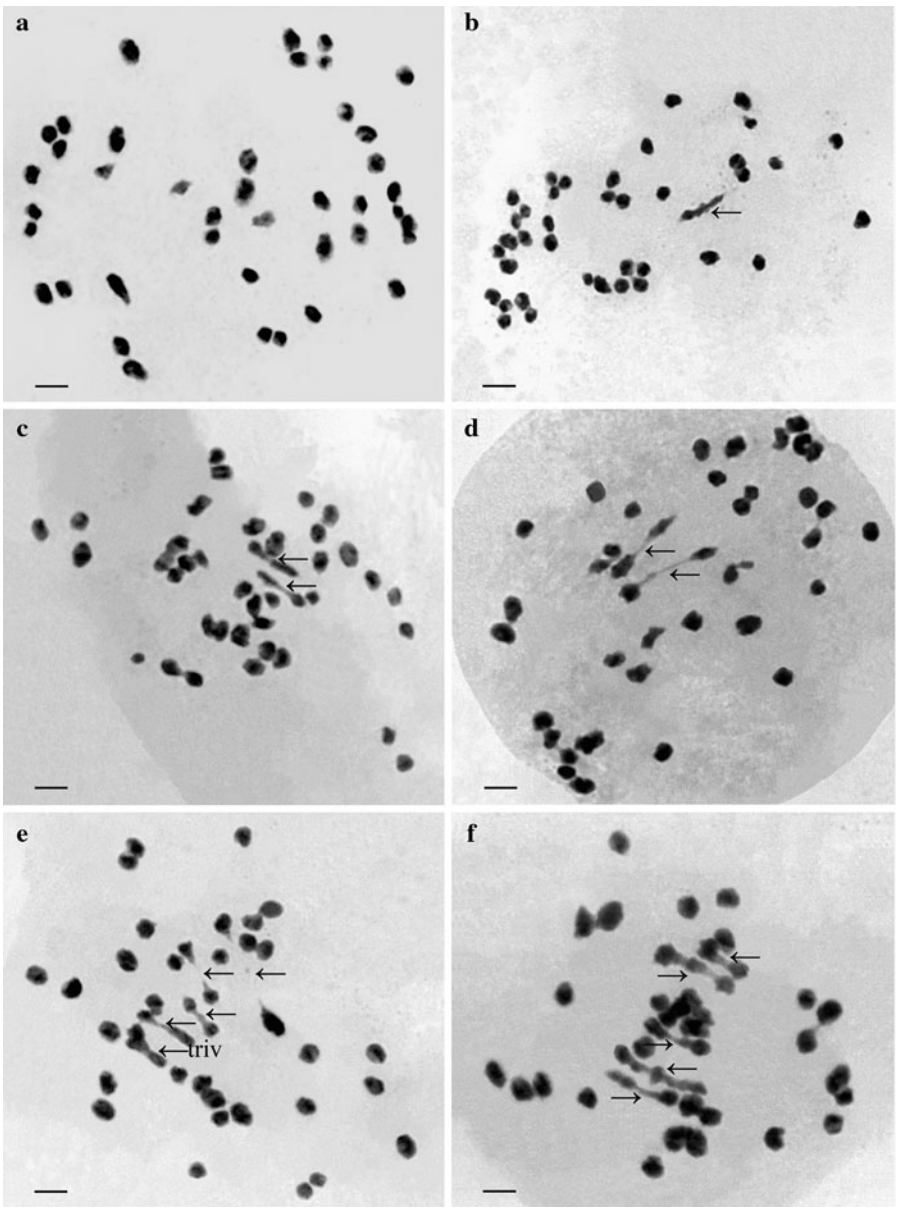

Fig. 2 Metaphase I in F_1 hybrid *N. wuttkei* × *N. tabacum* cv. Wiślica: **a** 40 I, **b** 1 II, 38 I **c**, **d** 2 II, 36 I, **e** 1 III, 4 II, 29 I—one of bivalents weakly conjugated **f** 5 II, 30 I. *Bar*: 5 μm; bivalents and trivalent marked by *arrows*

Table 3 Microspores produced in *N. wuttkei* × *N. tabacum* amphihaploid hybrid at the tetrad stage

Hybrids	Number of PMC's analyzed	PMC's observed (%)					
		Monads	Dyads	Tryads	Tetrads	Pentads	Micronuclei
N. wuttkei × *N. tabacum* cv. Puławski	260	5 (1.9)	10 (3.8)	15 (5.8)	211 (81.2)	19 (7.3)	29 (11.2)
N. wuttkei × *N. tabacum* cv. Wiślica	208	7 (3.4)	8 (3.8)	6 (2.9)	174 (83.7)	13 (6.3)	18 (8.7)
Total	468	12 (2.6)	18 (3.8)	21 (4.5)	385 (82.3)	32 (6.8)	47 (10.0)

Percentages of the total number in parentheses

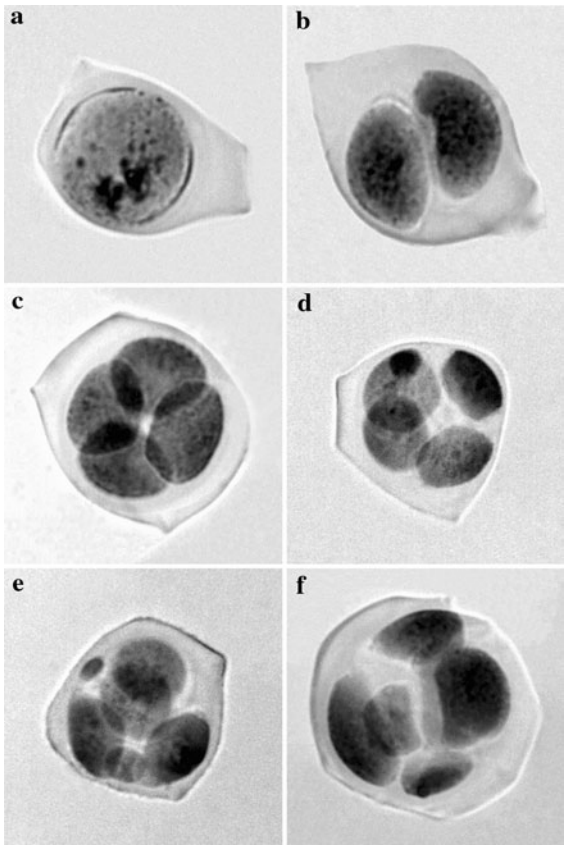

Fig. 3 Tetrad stage in F_1 *N. wuttkei* × *N. tabacum* cv. Wiślica: **a** monad, **b** dyad, **c** normal tetrad, **d** tetrad with 1 micronuclei, **e** tetrad with 2 micronuclei, **f** pentad

1991) and as far as we know, no report on the use of this species in tobacco breeding has yet been published. Likewise, the amphihaploid hybrid *N. wuttkei* × *N. tabacum* is a new combination of *Nicotiana* genomes, not reported heretofore.

Somatic chromosome numbers and their metaphase I pairing patterns clearly show that the 40-chromosome plants produced here were hybrids and amphihaploid, combining single haploid chromosome sets from each parent. These hybrids were produced only when *N. wuttkei* was used as the female parent. Such unilateral incompatibility frequently occurs in the conventional sexual interspecific crosses in *Nicotiana*, particularly when *N. tabacum* and a member of the section *Suaveolentes* are involved (Goodspeed 1954; De Verna et al.1987; Swaminathan and Murty 1957). The hybrids between *N. wuttkei* and three cultivars of *N. tabacum* used as male parents yielded filled capsules and the seeds germinated well, but nearly all seedlings died in the cotyledon stage. This is defined as "type II lethality" according to Yamada et al. (1999). Such phenomenon is frequent in the *Nicotianas* and is characteristic, among others, of reciprocal crosses between *N. tabacum* and many species from section *Suaveolentes* (Tezuka et al. 2010). In vitro culture allowed production of viable *N. wuttkei* × *N. tabacum* hybrids, albeit with a low success rate. In several hybrid combinations in the genus *Nicotiana,* the problem of seedling mortality can be successfully overcome by an in vitro culture of cotyledons (Lloyd 1975; Doroszewska and Berbeć 1996; Ternovski et al. 1974). Gerstel et al. (1979) and Inoue et al. (1996) suggested that genetic factors are responsible for hybrid lethality. According to Marubashi and Onosato (2002) cotyledon culture applied at first by Lloyd (1975) to the hybrid *N. suaveolens* × *N. tabacum* might induce mutation in a gene or genes involved in lethality.

All viable amphihaploid plants in this study were phenotypically uniform and intermediate between the parent species for several morphological traits. This is typical for other hybrids of *N. tabacum* with the members of the section *Suaveolentes* (Kostoff 1943; Goodspeed 1954; Gerstel et al. 1979; De Verna et al. 1987). Some investigators dealing with interspecific

Nicotiana hybrids reported departures from the amphihaploid chromosome number (Gerstel et al. 1979; Berbeć and Doroszewska 1981), and others recorded the expected number of somatic chromosomes (Doroszewska and Berbeć 1996; De Verna et al. 1987). Kostoff (1943) found that also unviable seedlings *N. suaveolens* × *N. tabacum* had the expected number of 40 somatic chromosomes.

In the present study there was no appreciable effect of the genotype of *N. tabacum* (cv. Puławski vs. cv. Wiślica) on chromosome pairing in the F_1 hybrid. In some other studies (Doroszewska and Berbeć 1996; Williams and Pandey 1974), mean number of bivalents in the amphihaploids differed depending on the *N. tabacum* cultivar used as the male parent.

A number of bivalents ranging from 0 to 5, with the modal number of 0, places the hybrid *N. wuttkei* × *N. tabacum* in the "minimum" pairing category according to Goodspeed (1954). Such a low degree of pairing is in close agreement with the data of Kostoff (1943) for an analogical hybrid involving *N. tabacum* and *N. suaveolens* ($2n = 32$) and this may be an additional justification for including *N. wuttkei* in the section *Suaveolentes*. Substantial variation for the bivalent number is a common phenomenon among F_1 hybrids in *Nicotiana*. The reason is the formation of loosely paired associations (either true bivalents or not) by chromosomes with a low degree of homology (Goodspeed 1954; Takenaka 1962). Some of these associations are broken by premature disjunction. A low rate of associations of higher valencies in metaphase I as well as few chromatid bridges between the separating chromosomes in later meiotic stages may indicate a low rate of crossover events in these hybrids.

There is likelihood that at least some of the bivalents observed in the *N. wuttkei* × *N. tabacum* amphihaploids, are autosyndetic (i.e. between the two subgenomes of *N. wuttkei* or *N. tabacum*) rather than allosyndetic (i.e. between the genomes of the parental species). This possibility is supported by findings by Collins and Sadasivaiah (1972) and by Takenaka and Tanaka (1956) who reported up to five bivalents in haploids of *N. tabacum*, clearlt a result of autosyndesis.

Fairly regular formation of tetrads in the hybrid *N. wuttkei* × *N. tabacum* is in agreement with observations of Sficas (1963), who found a tendency toward an equal, rather than random, distribution of unpaired chromosomes to the poles in *Nicotiana* hybrids. A possible explanation for this is that some chromosomes tend to forming achiasmate associations and then move to opposite poles. Formation of restitution nuclei (dyads and monads) by F_1 hybrids in *Nicotiana* is a regular phenomenon, apparently as a result of abnormal meiosis (Nikova and Zagorska 1990; Clayton 1950; Doroszewska and Berbec 1996). The hybrid *N. wuttkei* × *N. tabacum* was no exception in this respect. However, the relatively high rate of unreduced gametes produced by that hybrid did not correlate with its ability to produce viable pollen. Absence of viable pollen in the F_1 hybrids of *Nicotiana* is a rule rather than an exception (Ternovski et al. 1974; De Verna et al. 1987) although hybrids of *N. tabacum* with other members of the section *Suaveolentes* did show some vestigial pollen fertility (Doroszewska and Berbeć 1996; Clayton 1950; Kostoff 1943). The failure of the restitution microspores (nuclei) in the *N. wuttkei* × *N. tabacum* hybrids to develop into viable pollen could be a result of structural differences between the chromosomes resulting in gene imbalance. Other F_1 hybrids of *N. tabacum* with the members of the section *Suaveolentes* even if capable of producing partially fertile pollen were generally self- and cross-sterile (Kostoff 1943; Goodspeed 1954; Takenaka 1962; Wark 1970; Ternovski et al. 1974; De Verna et al. 1987; Doroszewska and Berbeć 1996).

In conclusion, sexual hybridization between *N. wuttkei* and *N. tabacum* is feasible although appears limited to a cross in one direction. As the success of transferring desirable traits from a wild into cultivated species largely depends on the pairing affinity of their chromosomes and thus on the potential for chromosome recombination, the opportunities for genetic exchange in this hybrid seem to be limited. Limited or otherwise, those opportunities can be explored only after restoring fertility to the hybrid since the directly produced amphihaploids of *N. wuttkei* × *N. tabacum* were completely self and cross sterile.

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